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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification: C07H 21/04, C12Q 1/68
A1
(11) International Publication Number: WO 00/69882
(43) International Publication Date: 23 November 2000 (23.11.2000)

(21) International Application Number: PCT/US00/13168
(22) International Filing Date: 15 May 2000 (15.05.2000)
(30) Priority Data:
60/134,180 13 May 1999 (13.05.1999) US
(60) Parent Application or Grant
THE PENN STATE RESEARCH FOUNDATION [/];
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Published

(54) Title: GENETIC MARKER FOR MEAT QUALITY, GROWTH, CARCASS AND REPRODUCTIVE TRAITS IN LIVESTOCK
(54) Titre: MARQUEUR GENETIQUE POUR LA QUALITE DE VIANDE, LA CROISSANCE, LA CARCASSE ET LES CARACTERISTIQUES REPRODUCTRICES DU BETAIL

(57) Abstract

Compositions and methods are provided for identifying polymorphisms associated with growth and reproductive traits in livestock.

(57) Abrégé

L'invention concerne des compositions et des procédés permettant l'identification des polymorphismes associés à la croissance et aux caractéristiques de reproduction du bétail.

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60/134,180 13 May 1999 (13.05.99) US

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(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

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Description

GENETIC MARKER FOR MEAT QUALITY, GROWTH, CARCASS AND REPRODUCTIVE TRAITS IN LIVESTOCK

FIELD OF THE INVENTION

This invention relates generally to the detection of genetic differences associated with growth, body composition and reproductive traits among livestock. More specifically, the invention provides compositions and methods for predicting heritability of certain traits related to steroid biosynthesis and metabolism.

BACKGROUND OF THE INVENTION

Several publications are referenced in this application by author name, year and journal of publication in parentheses in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these publications is incorporated by reference herein.

Steroid hormones play a crucial role in the differentiation, development, growth and physiological function of most animal tissues. The first and rate-limiting step in the biosynthesis of all steroid hormones is the conversion of cholesterol into pregnenolone by the cholesterol side chain cleavage enzyme p450scc. The gene which encodes P450scc is termed CYP11a1. Cytochromes P450 are a diverse group of heme-containing mono-oxygenases (termed CYP's; see Nelson et al., DNA Cell Biol. (1993) 12: 1-51) that catalyze a variety of oxidative conversions, notably of steroids but also of fatty acids and xenobiotics. CYP's are most abundantly expressed in the testis, ovary, placenta, adrenal glands and liver. In the reproductive organs, such as testis, ovary and placenta, the most

important steroid hormones produced are the androgens (e.g., testosterone), the estrogens (e.g., estradiol) and progestins (e.g., progesterone). In the adrenal glands, the most important steroids are the mineralcorticoids (e.g., aldosterone) and the glucocorticoids (e.g., cortisol).

The frequent occurrence of off-odors or off-tastes in cooked pork from boars, commonly known as "boar odor" or "boar taint", is the primary reason for the common practice of castration in swine production. 5α-androstenone (5α-androst-16-en-3-one), an important compound responsible for boar taint, is synthesized in the boar testis along with other 16-androstene steroids, androgens, and estrogens. At puberty, testicular production of Δ16-androstenes, in particular 5α-androstenone (androstenone), increases sharply. This results in the accumulation of androstenone in various body compartments, notably in fat deposits throughout the body and in the submaxillary salivary gland (SMG), where there is a specific binding protein for Δ16-androstenes. Concentration of androstenone and other Δ16-androstenes in the SMG are highly correlated with concentrations of Δ16- androstenes in the fat. Measurement of Δ16-androstenes in the SMG is used, in fact, as a test method to determine the presence or absence of boar taint. Thus, due to this increase in Δ16-androstenes, it is common in the industry to castrate the young male boars to minimize this taint in the meat. However, if the problem of boar taint were overcome, raising boars rather than raising castrates (barrows) for pork would have considerable economic advantages. Although boars and barrows gain weight at equivalent rates, boars produce carcasses containing 20-30% less fat. Thus, boars are much more efficient at producing lean muscle. In addition, boars utilize feed

more efficiently than barrows (10% less feed consumed per unit of body weight). Since feed represents the major cost in swine production, raising boars for pork would have significant economic advantages.

In the United States, approximately 90 million hogs are slaughtered annually with an approximate value of $11 billion. Feed accounts for the major portion of the costs of swine production, accounting for roughly 70% of production costs. Thus, a 10% improvement in feed efficiency would produce savings of 7% of the total cost of production. On a nation-wide basis, considering male swine only, this translates to total market savings of $335 million. The loss of production efficiency caused by the practice of castration represents a very large economic loss to the swine industry throughout the world.

Identification of the inheritance pattern(s) and genetic bases for alterations in steroid biosynthesis in livestock has utility in the production of meat, dairy and egg products of higher quality. It is an object of the present invention to provide compositions and methods for identifying such genetic alterations.

SUMMARY OF THE INVENTION

In accordance with the present invention, methods for identifying genetic alterations associated with steroid biosynthesis are provided. In one embodiment of the invention, the presence or absence of a polymorphic marker in the CYP11a1 DNA of a test subject is determined. Such test subjects are selected from important livestock species, including without limitation, pigs, cows, chickens and sheep. In accordance with the present invention, it has been determined that certain polymorphisms in the CYP11a1 gene are associated with increased growth, reproductive

and carcass traits. Thus, screening methods are provided for identifying those test subjects which possess these beneficial CYP11a1 alleles. Identification of such livestock facilitates the implementation of breeding programs for developing stock having these improved genetic traits.

As is well known to those of skill in the art, a variety of techniques may be utilized when comparing nucleic acid molecules for sequence differences. These include by way of example, restriction fragment length polymorphism analysis, heteroduplex analysis, single strand conformation polymorphism analysis, denaturing gradient electrophoresis and temperature gradient electrophoresis.

In a preferred embodiment of the invention, the CYP11a1 polymorphism is a restriction fragment polymorphism and the assay comprises identifying the CYP11a1 gene from genetic material isolated from the test subject; exposing the gene to a restriction enzyme that yields restriction fragments of the gene of varying length; separating the restriction fragments to form a restriction pattern, such as by electrophoresis or HPLC separation; and comparing the resulting restriction fragment pattern from a test subject CYP11a1 gene that is either known to have or not to have the desired marker. If a test subject tests positive for the marker, such a subject can be considered for inclusion in the breeding program. If the test subject does not test positive for the marker genotype, the test subject can be culled from the group and otherwise used.

In a particularly preferred embodiment, the test subject is a pig, the polymorphism is in the 5'UTR of the CYP11a1 gene and the restriction enzyme is SphI. Thus, in this aspect, it is an object of the invention to provide a method of screening pigs to determine those

more likely to have decreased testis weight and reduced boar taint, longer carcasses, improved rate of gain, or heavier weaning weights when bred to or to select against pigs which have alleles indicating larger testis size, increased boar taint, reduced carcass length, lower rate of gain, or lighter weaning weights. As used herein "smaller testis size" means a significant decrease in testis size below the mean for a given population. As used herein "reduced boar taint" means a significant decrease in boar taint below the mean for a given population. As used herein "increased carcass length" means a significant increase in carcass length above the mean for a given population. As used herein "higher rate of gain" means a significant increase in rate of gain above the mean for a given population. As used herein "heavier weaning weights" mean an increase in weaning weight above the mean for a given population. The method of the invention comprises the steps: 1) obtaining a sample of genomic DNA from a pig; and 2) analyzing the genomic DNA obtained in 1) to determine which CYP11a1 allele(s) is/are present. Briefly, a sample of genetic material is obtained from a pig, and the sample is analyzed to determine the presence or absence of a polymorphism in the CYP11a1 gene that is correlated with reduced boar taint, smaller testis size, increased carcass length, higher rate of gain, and/or increased weaning weight.

In a most preferred embodiment the gene is isolated by the used of primers and DNA polymerase to amplify a specific region of the gene which contains the polymorphism. Next the amplified region is digested with a restriction enzyme and fragments are separated. Visualization of the RFLP pattern is by simple staining of the fragments, or by labeling the primers or the nucleoside triphosphates used in amplification.

In another embodiment, the invention comprises a method for identifying a genetic marker for boar taint, testis size, carcass length, rate of gain, and/or weaning weight in a particular population. Male and female pigs of the same breed or breed cross or similar genetic lineage are bred, and traits such as boar taint, testis size, carcass length, rate of gain, and/or weaning weight are determined. A polymorphism in the CYP11a1 gene of each pig is identified and associated with the traits of boar taint, testis size, carcass length, rate of gain, and/or weaning weight. Preferably, RFLP analysis is used to determine the polymorphism, and most preferably, the DNA is digested with the restriction endonuclease SphI, or other restriction endonuclease that differentially cleaves the restriction site based on the presence or absence of the polymorphism.

Methods are also provided to establish linkage between specific alleles of alternative DNA markers and alleles of DNA markers known to be associated with a particular gene (e.g. the CYP11a1 gene discussed herein), which have been previously shown to be associated with a particular trait. Thus, selection for pigs likely to have reduced boar taint, smaller testes, increased carcass length, higher rate of gain, and/or heavier weaning weights, or alternatively to select against pigs likely to have increased boar taint, larger testes, reduced carcass length, lower rate of gain, and/or lighter weaning weights, may be done indirectly, by selecting for certain alleles of a CYP11a1 associated marker through the selection of specific alleles of alternative markers located on the same chromosome as CYP11a1.

The invention further comprises kits for evaluating a sample of test subject DNA for the presence in test

subject genetic material of a desired marker located in the test subject, CYP11a1 gene indicative of the inheritable traits of boar taint (in the pig), testis size, carcass length, rate of gain, and/or weaning weight. At a minimum, using the pig as the test subject, the kit is a container with one or more reagents that identify a polymorphism in the pig CYP11a1 gene. Preferably, the reagent is a set of oligonucleotide primers capable of amplifying a fragment of the pig CYP11a1 gene that contains the polymorphism. More preferably, the kit further contains a restriction enzyme that cleaves the pig CYP11a1 gene in at least one place. In a most preferred embodiment the restriction enzyme is SphI or one which cuts at the same recognition site.

The following definitions are provided to facilitate an understanding of the present invention:

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA". Hybridization probes may be DNA or RNA, or any synthetic nucleotide structure capable of binding in a base-specific manner to a complementary strand of nucleic acid. For example, probes include peptide nucleic acids, as described in Nielsen et al., Science 254:1497-1500 (1991).

"Linkage" describes the tendency of genes, alleles, loci or genetic markers to be inherited together as a result of their location on the same chromosome, and is measured by percent recombination (also called recombination fraction, or θ) between the two genes, alleles, loci or genetic markers. The closer two loci physically are on the chromosome, the lower the recombination fraction will be. Normally, when a polymorphic site from within a disease-causing gene is tested for linkage with the disease, the recombination fraction will be zero, indicating that the disease and the disease-causing gene are always co-inherited. In rare cases, when a gene spans a very large segment of the genome, it may be possible to observe recombination between polymorphic sites on one end of the gene and causitive mutations on the other. However, if the causative mutation is the polymorphism being tested for linkage with the disease, no recombination will be observed.

"Centimorgan" is a unit of genetic distance signifying linkage between two genetic markers, alleles, genes or loci, corresponding to a probability of recombination between the two markers or loci of 1% for any meiotic event.

"Linkage disequilibrium" or "allelic association" means the preferential association of a particular allele, locus, gene or genetic marker with a specific allele, locus, gene or genetic marker at a nearby chromosomal location more frequently than expected by chance for any particular allele frequency in the population.

An "oligonucleotide" can be DNA or RNA, and single-

or double-stranded. Oligonucleotides can be naturally occurring or synthetic, but are typically prepared by synthetic means.

The term "primer" refers to an oligonucleotide capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligonucleotide. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 15 to 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to hybridize with a template. The term "primer" may refer to more than one primer, particularly in the case where there is some ambiguity in the information regarding one or both ends of the target region to be amplified. For instance, if a region shows significant levels of polymorphism or mutation in a population, mixtures of primers can be prepared that will amplify alternate sequences. A primer can be labeled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in an ELISA), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available. A label can also be used to "capture" the

primer, so as to facilitate the immobilization of either the primer or a primer extension product, such as amplified DNA, on a solid support.

"Chromosome 7 set" in boars for example, means the two copies of chromosome 7 found in somatic cells or the one copy in germ line cells of a test subject or family member. The two copies of chromosome 7 may be the same or different at any particular allele, including alleles at or near the locus of interest. The chromosome 7 set may include portions of chromosome 7 collected in chromosome 7 libraries, such as plasmid, yeast, or phage libraries, as described in Sambrook et al., Molecular Cloning, 2nd Edition, and in Mandel et al., Science 258:103-108 (1992).

"Penetrance" is the percentage of individuals with a defective gene or polymorphism who show some symptoms of a trait resulting from that genetic alteration. Expressivity refers to the degree of expression of the trait (e.g., mild, moderate or severe).

"Polymorphism" refers to the occurrence of two or more genetically determined alternative sequences or alleles in a population. A polymorphic marker is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%. A polymorphic locus may be as small as one base pair difference. Polymorphic markers suitable for use in the invention include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, and other microsatellite sequences.

"Restriction fragment length polymorphism" (RFLP) means a variation in DNA sequence that alters the length of a restriction fragment as described in Botstein et al., Am. J. Hum. Genet. 32:314-331 (1980). The restriction fragment length polymorphism may create or delete a restriction site, thus changing the length of the restriction fragment. For example, the DNA sequence GAATTC are the six bases, together with its complementary strand CTTAAG which comprises the recognition and cleavage site of the restriction enzyme EcoRI. Replacement of any of the six nucleotides on either strand of DNA to a different nucleotide destroys the EcoRI site. This RFLP can be detected by, for example, amplification of a target sequence including the polymorphism, digestion of the amplified sequence with EcoRI, and size fractionation of the reaction products on an agarose or acrylamide gel. If the only EcoRI restriction enzyme site within the amplified sequence is the polymorphic site, the target sequences comprising the restriction site will show two fragments of predetermined size, based on the length of the amplified sequence. Target sequences without the restriction enzyme site will only show one fragment, of the length of the amplified sequence. Similarly, the RFLP can be detected by probing an EcoRI digest of Southern blotted DNA with a probe from a nearby region such that the presence or absence of the appropriately sized EcoRI fragment may be observed. RFLP's may be caused by point mutations which create or destroy a restriction enzyme site, VNTR's, dinucleotide repeats, deletions, duplications, or any other sequence-based variation that creates or deletes a restriction enzyme site, or alters the size of a restriction fragment.

"Variable number of tandem repeats" (VNTR's) are

short sequences of nucleic acids arranged in a head to tail fashion in a tandem array, and found in each individual, as described in Wyman et al., Proc. Nat. Acad. Sci. 77:6754-6758 (1980). Generally, the VNTR sequences are comprised of a core sequence of at least 16 base pairs, with a variable number of repeats of that sequence. Additionally, there may be variation within the core sequence, Jefferys et al., Nature 314:67-72 (1985). These sequences are highly individual, and perhaps unique to each individual. Thus, VNTR's may generate restriction fragment length polymorphisms, and may additionally serve as size-based amplification product differentiation markers.

"Microsatellite sequences" comprise segments of at least about 10 base pairs of DNA consisting of a variable number of tandem repeats of short (1-6 base pairs) sequences of DNA(Clemens et al., Am. J. Hum. Genet. 49:951-960 1991). Microsatellite sequences are generally spread throughout the chromosomal DNA of an individual. The number of repeats in any particular tandem array varies greatly from individual to individual, and thus, microsatellite sequences may serve to generate restriction fragment length polymorphisms, and may additionally serve as size-based amplification product differentiation markers.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the sequence of approximately 630 base pairs of the 5'untranslated region of the porcine CYP11A1 gene (SEQ ID NO: 1). The PCR fragment was produced using DNA extracted from porcine testis samples. The primers used were forward primer (SEQ ID NO:2) and reverse primer (SEQ ID NO:3).

Figure 2 depicts the polymorphic pattern of SphI digested PCR product. The forward and reverse primers were used in the following PCR conditions: Two minutes @ 94°C, 35 cycles of one minute @ 94°C, one minute @ 55°C, one minute @ 72°C and a final two minutes @ 72°C.. Samples were digested with SphI (New England Biolabs) and separated on 1.5% agarose gel at 50 volts for 45 minutes at room temperature. Gels were stained with ethidium bromide. Lane 1: low molecular weight markers; Lane 2: undigested PCR fragment; Lanes 3 and 7: genotype CT; and Lanes 4-6: genotype CC. A Restriction Fragment Length Polymorphism (RFLP) was discovered whereby the 630 bp PCR fragment from CC pigs was digested into a 450 bp product while the PCR fragment from the CT pigs was only partially digested, which indicates the presence of the T allele.

Figure 3 depicts the concentrations of submaxillary salivary gland (SMG) Δ-16 androstenes in boars of the CC versus the CT genotype. Five out of thirty of the CC boars exhibited SMG Δ-16 androstene concentrations greater than the recommended threshold level for identifying tainted carcasses (55 µg/g SMG). All of boars carrying the T allele (n=20) were below the recommended threshold level for boar taint.

Figure 4 is a table that shows the observed differences in various growth, carcass, and reproductive traits of CC versus CT boars. The greater weights of testes, submaxillary glands and bulbourethral glands, as well as higher concentrations of SMG Δ-16-androstenes, are all indications of higher boar taint in the CC boars. Surprisingly the CC boars also had 5.9% increase in rate of gain and longer carcasses as well.

Figure 5 shows the sequence of the bovine CYP11a1 gene, including 948 nucleotide of the 5' UTR.

Figure 6 shows the sequence of the chicken CYP11a1 gene, including 137 nucleotide of the 5'UTR.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, materials and methods are provided for diagnosing genetic alterations in the CYP11a1 gene associated with aberrant or increased sterioid biosynthesis in livestock. In the mouse, polymorphic variation in CYP11a1 is responsible for genetic differences in testosterone production. In mouse, CYP11a1 maps to chromosome 9. This region is syntenic with porcine chromosome 7.

A principle cause of taint in the boar is the presence of the Δ-16 steroid, androstenone, which is one of many steroids produced in the boar testis. Androstenone and androstenone metabolites such as androstenol are secreted by the testis and sequestered in the submaxillary salivary glands (SMG). During mating behavior these steroids are released into the air through the saliva and function as sexual pheromones whereby they induce estrous behavior in female pigs (sows). Since Δ-16 steroids are highly lipophilic, androstenone is also stored in body fat, where its presence in high concentrations contributes to the off-flavors in pork known as boar taint.

Concentrations of androstenone in the fat are highly heritable. A quantitative trait locus (QTL) has been identified for fat androstenone (microsatellite marker SO102), which is located on porcine chromosome 7 in the region of the swine leukocyte antigen complex (SLA). In accordance with the present invention, a particular genetic polymorphic sequence has been

identified which is associated with androstenone production and boar taint.

The presence of a quantitative trait locus (QTL) for fat androstenone on chromosome 7 in the pig suggests that porcine CYP11a1 may be located on chromosome 7 and, as the rate limiting enzyme in steroid synthesis may be an important control point for androsterone synthesis and the occurrence of boar taint.

A genomic search was conducted to compare 2.4 kb of the untranslated region (5`UTR) of the porcine CYP11a1 gene from a preselected group of boars in order to determine if polymorphisms exist which are associated with compounds which cause boar taint. First, comparisons of the genotypes of five "high taint" and five "low taint" boars by direct sequencing of PCR products (using the ABI Prism 377 at the Nucleic Acid Facility, Penn State University Biotechnology Institute) revealed the presence of one single nucleotide polymorphism (SNP) in the entire 2.4 kb 5' UTR. This SNP (CT allele) was discovered only in boars that exhibited low concentrations of delta-16 steroids in the salivary gland, a measurement that is highly correlated with androstenone concentrations in the fat. This polymorphism consists of either a thymidine (T) or a cytosine (C) at position - 155 from the start site of translation. The polymorphism was located in a restriction enzyme recognition site such that the presence of the T allele would change the restriction fragment length pattern observed after digestion with specific restriction enzymes. In this particular case, the restriction enzyme used was SphI (New England Biolabs). Additional restriction enzymes are available which are able to cut the same DNA sequence. Presence or absence of the T allele was determined by examination of restriction digests of CYP11a1 5'UTR using SphI.

Presence of the T allele, either homozygous (TT) or heterozygous (CT), was associated with low boar taint. Presence of the CC allele was associated with high boar taint, as well as with increased testis weight, bulbourethral gland length and weight and submaxillary salivary gland weights. In addition, boars that possessed the CC allele exhibited a 5.9% improvement in rate of gain as well as longer carcasses.

The discovery that this polymorphism is associated with increased rate of gain and carcass length in addition to its effects on reproductive traits indicates that this polymorphism affects many other growth and developmental traits. Thus, presence or absence of this polymorphism may also be associated with feed efficiency and with birth weight. The association of this polymorphism with reproductive traits such as testis weight, bulbourethral gland length and weight, submaxillary gland weight, and Δ-16 steroid concentrations, are all indications of a general effect on gonadal steroid production.

The data presented herein indicate that the presence or absence of the CYP11a1 polymorphism may have effects on other reproductive traits such as ovulation rate, litter size, milk production, and fertility (both male and female). Additionally, since the adrenal gland is another site where CYP11a1 is expressed to produce glucocorticoid steroids such cortisol, this polymorphism may be associated with disease response traits since these traits are known to be modulated by adrenal steroids.

In a further aspect of the invention, this genetic marker may also be used in combination with other genetic markers to produce favorable combinations of alleles or to select against those test subjects carrying unfavorable combinations. Examples of some of

these previously identified genes are: tumor necrosis factor alpha (TNFa), CYP11a1, prolactin (PRL), estrogen receptor (ER) and prolactin receptor (PRIR). Examples of some of these previously identified microsatellite markers are: S0064, S0102, S0078, S0158, S0066, SW304, SW1083, S0101, and S0212.

Additional polymorphisms in the porcine CYP11a1 gene may be identified using the methods of the present invention. Such alterations may occur in the untranslated region of the gene but may also be identified in the translated region, as well as in the intronic and exonic sequences. It is likely that a subset of these changes will cause or be associated with changes in androgen function and phenotypic traits. Once such genetic alterations are identified, it is possible to introduce these or similar changes into the genome by known techniques in order to produce transgenic animals that possess a desired CYP11a1 genotype. The data further suggest that polymorphisms in homologous areas of CYP11a1 of other agriculturally important species are likely to cause or be associated with similar changes in function and phenotype.

In a further aspect of the invention, the corresponding CYP11a1 sequences from the cow and the chicken are provided. This information facilitates genomic scanning of the 5'UTR of the bovine or chicken CYP11a1 to reveal polymorphisms that are associated with growth, carcass traits, and reproduction (including milk production and egg production).

DIAGNOSTIC KITS FOR PRACTICING THE METHODS OF THE INVENTION

The present invention also includes kits for the practice of the methods of the invention. The kits comprise a vial, tube, or any other container which

contains one or more oligonucleotides, which hybridizes to a DNA segment which DNA segment which is or is linked to the CYP11a1 gene. Some kits contain two such oligonucleotides, which serve as primers to amplify a segment of chromosome DNA. The segment selected for amplification can be a CYP11a1 gene that includes a site at which a variation is known to occur. Some kits contain a pair of oligonucleotides for detecting precharacterized variations. For example, some kits contain oligonucleotides suitable for allele-specific oligonucleotide hybridization, or allele-specific amplification hybridization. The kits of the invention may also contain components of the amplification system, including PCR reaction materials such as buffers and a thermostable polymerase. In other embodiments, the kit of the present invention can be used in conjunction with commercially available amplification kits, such as may be obtained from GIBCO BRL (Gaithersburg, Md.) Stratagene (La Jolla, Calif.), Invitrogen (San Diego, Calif.), Schleicher & Schuell (Keene, N.H.), Boehringer Mannheim (Indianapolis, Ind.). The kits may optionally include positive or negative control reactions or markers, molecular weight size markers for gel electrophoresis, and the like. The kits usually include labeling or instructions indicating the suitability of the kits for diagnosing steroid biosynthesis alterations and indicating how the oligonucleotides are to be used for that purpose. The term "label" is used generically to encompass any written or recorded material that is attached to, or otherwise accompanies the diagnostic at any time during its manufacture, transport, sale or use.

MODES OF PRACTICING THE INVENTION

1. Linkage Analysis

Determining linkage between a polymorphic marker

and a locus associated with a particular phenotype is performed by mapping polymorphic markers and observing whether they co-segregate with the high taint phenotype (for example) on a chromosome in an informative meiosis. See, e.g., Kerem et al., Science 245:1073-1080 (1989); Monaco et al., Nature 316:842 (1985); Yamoka et al., Neurology 40:222-226 (1990), and as reviewed in Rossiter et al., FASEB Journal 5:21-27 (1991). A single pedigree rarely contains enough informative meioses to provide definitive linkage, because families are often small and markers may be not sufficiently informative. For example, a marker may not be polymorphic in a particular family.

Linkage may be established by an affected sib-pairs analysis as described in Terwilliger & Ott, Handbook of Human Genetic Linkage (Johns Hopkins, Md., 1994), Ch. 26. This approach requires no assumptions to be made concerning penetrance or variant frequency, but only takes into account the data of a relatively small proportion (i.e., the SIB pairs) of all the family members whose phenotype and polymorphic markers have been determined. Specifically, the affected SIB pairs analysis scores each pair of affected SIBS as sharing (concordant) or not sharing (discordant) the same allelic variant of each polymorphic marker. For each marker, a probability is then calculated that the observed ratio of concordant to discordant SIB pairs would arise without linkage of the marker.

As described in Thompson & Thompson, Genetics in Medicine, 5th ed, 1991, W.B. Saunders Company, Philadelphia, in linkage analysis, one calculates a series of likelihood ratios (relative odds) at various possible values of θ, ranging from $\theta = 0.0$ (no recombination) to $\theta = 0.50$ (random assortment). Thus, the likelihood ratio at a given value of θ is (likelihood of

data if αloci are linked at θ)/(likelihood of data if loci are unlinked). Evidence in support of linkage is usually expressed as the $\log_{10}$ of this ratio and called a "lod score" for "logarithm of the odds." For example, a lod score of 5 indicates 100,000:1 odds that the linkage being observed did not occur by chance.

The use of logarithms allows data collected from different families to be combined by simple addition. Computer programs are available for the calculation of lod scores for differing values of θ. Available programs include LIPED, and MLINK (Lathrop, Proc. Nat. Acad. Sci. 81:3443-3446 (1984).

For any particular lod score, a recombination fraction may be determined from mathematical tables. See Smith et al., Mathematical tables for research workers in human genetics (Churchill, London, 1961) and Smith, Ann. Hum. Genet. 32:127-150 (1968). The value of θ at which the lod score is the highest is considered to be the best estimate of the recombination fraction, the "maximum likelihood estimate".

Positive lod score values suggest that the two loci are linked, whereas negative values suggest that linkage is less likely (at that value of θ) than the possibility that the two loci are unlinked. By convention, a combined lod score of +3 or greater (equivalent to greater than 1000:1 odds in favor of linkage) is considered definitive evidence that two loci are linked. Similarly, by convention, a negative lod score of -2 or less is taken as definitive evidence against linkage of the two loci being compared. If there are sufficient negative linkage data, a locus can be excluded from an entire chromosome, or a portion thereof, a process referred to as exclusion mapping. The search is then focused on the remaining non-excluded chromosomal locations. For a general discussion of lod scores and

linkage analysis, see, e.g., T. Strachan, Chapter 4, "Mapping the human genome" in The Human Genome, 1992 BIOS Scientific Publishers Ltd. Oxford.

The data can also be subjected to haplotype analysis. This analysis assigns allelic markers between the chromosomes of an individual such that the number of recombinational events needed to account for segregation between generations is minimized. Linkage may also be established by determining the relative likelihood of obtaining observed segregation data for any two markers when the two markers are located at a recombination fraction θ, versus the situation in which the two markers are not linked, and thus segregating independently.

2. Isolation and Amplification of DNA

Samples of patient, proband, test subject, or family member genomic DNA are isolated from any convenient source including saliva, buccal cells, hair roots, blood, cord blood, amniotic fluid, interstitial fluid, peritoneal fluid, chorionic villus, and any other suitable cell or tissue sample with intact interphase nuclei or metaphase cells. The cells can be obtained from solid tissue as from a fresh or preserved organ or from a tissue sample or biopsy. The sample can contain compounds which are not naturally intermixed with the biological material such as preservatives, anticoagulants, buffers, fixatives, nutrients, antibiotics, or the like.

Methods for isolation of genomic DNA from these various sources are described in, for example, Kirby, DNA Fingerprinting, An Introduction, W.H. Freeman & Co. New York (1992). Genomic DNA can also be isolated from cultured primary or secondary cell cultures or from

transformed cell lines derived from any of the aforementioned tissue samples.

Samples of patient, proband, test subject or family member RNA can also be used. RNA can be isolated from tissues expressing the CYP11a1 gene as described in Sambrook et al., supra. RNA can be total cellular RNA, mRNA, poly A+ RNA, or any combination thereof. For best results, the RNA is purified, but can also be unpurified cytoplasmic RNA. RNA can be reverse transcribed to form DNA which is then used as the amplification template, such that the PCR indirectly amplifies a specific population of RNA transcripts. See, e.g., Sambrook, supra, Kawasaki et al., Chapter 8 in PCR Technology, (1992) supra, and Berg et al., Hum. Genet. 85:655-658 (1990).

3. PCR Amplification

The most common means for amplification is polymerase chain reaction (PCR), as described in U.S. Pat. Nos. 4,683,195, 4,683,202, 4,965,188 each of which is hereby incorporated by reference. If PCR is used to amplify the target regions in blood cells, heparinized whole blood should be drawn in a sealed vacuum tube kept separated from other samples and handled with clean gloves. For best results, blood should be processed immediately after collection; if this is impossible, it should be kept in a sealed container at 4° C until use. Cells in other physiological fluids may also be assayed. When using any of these fluids, the cells in the fluid should be separated from the fluid component by centrifugation.

Tissues should be roughly minced using a sterile, disposable scalpel and a sterile needle (or two scalpels) in a 5 mm Petri dish. Procedures for removing

paraffin from tissue sections are described in a variety of specialized handbooks well known to those skilled in the art.

To amplify a target nucleic acid sequence in a sample by PCR, the sequence must be accessible to the components of the amplification system. One method of isolating target DNA is crude extraction which is useful for relatively large samples. Briefly, mononuclear cells from samples of blood, amniocytes from amniotic fluid, cultured chorionic villus cells, or the like are isolated by layering on sterile Ficoll-Hypaque gradient by standard procedures. Interphase cells are collected and washed three times in sterile phosphate buffered saline before DNA extraction. If testing DNA from peripheral blood lymphocytes, an osmotic shock (treatment of the pellet for 10 sec with distilled water) is suggested, followed by two additional washings if residual red blood cells are visible following the initial washes. This will prevent the inhibitory effect of the heme group carried by hemoglobin on the PCR reaction. If PCR testing is not performed immediately after sample collection, aliquots of 10^6 cells can be pelleted in sterile Eppendorf tubes and the dry pellet frozen at -20° C until use.

The cells are resuspended (10^6 nucleated cells per 100 µl) in a buffer of 50 mM Tris-HCl (pH 8.3), 50 mM KCl 1.5 mM $MgCl_2$, 0.5% Tween 20, 0.5% NP40 supplemented with 100 µg/ml of proteinase K. After incubating at 56° C for 2 hr, the cells are heated to 95° C for 10 min to inactivate the proteinase K and immediately moved to wet ice (snap-cool). If gross aggregates are present, another cycle of digestion in the same buffer should be undertaken. Ten µl of this extract is used for amplification.

When extracting DNA from tissues, e.g., chorionic

villus cells or confluent cultured cells, the amount of the above mentioned buffer with proteinase K may vary according to the size of the tissue sample. The extract is incubated for 4-10 hrs at 50°-60° C and then at 95° C for 10 minutes to inactivate the proteinase. During longer incubations, fresh proteinase K should be added after about 4 hr at the original concentration.

When the sample contains a small number of cells, extraction may be accomplished by methods as described in Higuchi, "Simple and Rapid Preparation of Samples for PCR", in PCR Technology, Ehrlich, H. A. (ed.), Stockton Press, New York, which is incorporated herein by reference. PCR can be employed to amplify target regions from chromosome 7 in very small numbers of cells (1000-5000) derived from individual colonies from bone marrow and peripheral blood cultures. The cells in the sample are suspended in 20 µl of PCR lysis buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP40, 0.45% Tween 20) and frozen until use. When PCR is to be performed, 0.6 µl of proteinase K (2 mg/ml) is added to the cells in the PCR lysis buffer. The sample is then heated to about 60° C and incubated for 1 hr. Digestion is stopped through inactivation of the proteinase K by heating the samples to 95° C for 10 min and then cooling on ice.

A relatively easy procedure for extracting DNA for PCR is a salting out procedure adapted from the method described by Miller et al., Nucleic Acids Res. 16:1215 (1988), which is incorporated herein by reference. Mononuclear cells are separated on a Ficoll-Hypaque gradient. The cells are resuspended in 3 ml of lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na_2 EDTA, pH 8.2). Fifty µl of a 20 mg/ml solution of proteinase K and 150 µl of a 20% SDS solution are added to the cells and then incubated at 37° C overnight. Rocking the tubes

during incubation will improve the digestion of the sample. If the proteinase K digestion is incomplete after overnight incubation (fragments are still visible), an additional 50 µl of the 20 mg/ml proteinase K solution is mixed in the solution and incubated for another night at 37° C on a gently rocking or rotating platform. Following adequate digestion, one ml of a 6M NaCl solution is added to the sample and vigorously mixed. The resulting solution is centrifuged for 15 minutes at 3000 rpm. The pellet contains the precipitated cellular proteins, while the supernatant contains the DNA. The supernatant is removed to a 15 ml tube that contains 4 ml of isopropanol. The contents of the tube are mixed gently until the water and the alcohol phases have mixed and a white DNA precipitate has formed. The DNA precipitate is removed and dipped in a solution of 70% ethanol and gently mixed. The DNA precipitate is removed from the ethanol and air-dried. The precipitate is placed in distilled water and dissolved.

Kits for the extraction of high-molecular weight DNA for PCR include a Genomic Isolation Kit A.S.A.P. (Boehringer Mannheim, Indianapolis, Ind.), Genomic DNA Isolation System (GIBCO BRL, Gaithersburg, Md.), Elu-Quik DNA Purification Kit (Schleicher & Schuell, Keene, N.H.), DNA Extraction Kit (Stratagene, La Jolla, Calif.), TurboGen Isolation Kit (Invitrogen, San Diego, Calif.), and the like. Use of these kits according to the manufacturer's instructions is generally acceptable for purification of DNA prior to practicing the methods of the present invention.

The concentration and purity of the extracted DNA can be determined by spectrophotometric analysis of the absorbance of a diluted aliquot at 260 nm and 280 nm. After extraction of the DNA, PCR amplification may

proceed. The first step of each cycle of the PCR involves the separation of the nucleic acid duplex formed by the primer extension. Once the strands are separated, the next step in PCR involves hybridizing the separated strands with primers that flank the target sequence. The primers are then extended to form complementary copies of the target strands. For successful PCR amplification, the primers are designed so that the position at which each primer hybridizes along a duplex sequence is such that an extension product synthesized from one primer, when separated from the template (complement), serves as a template for the extension of the other primer. The cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

In a particularly useful embodiment of PCR amplification, strand separation is achieved by heating the reaction to a sufficiently high temperature for an sufficient time to cause the denaturation of the duplex but not to cause an irreversible denaturation of the polymerase (see U.S. Pat. No. 4,965,188, incorporated herein by reference). Typical heat denaturation involves temperatures ranging from about 80° C to 105° C for times ranging from seconds to minutes. Strand separation, however, can be accomplished by any suitable denaturing method including physical, chemical, or enzymatic means. Strand separation may be induced by a helicase, for example, or an enzyme capable of exhibiting helicase activity. For example, the enzyme RecA has helicase activity in the presence of ATP. The reaction conditions suitable for strand separation by helicases are known in the art (see Kuhn Hoffman-Berling, 1978, CSH-Quantitative Biology, 43:63-67; and Radding, 1982, Ann. Rev. Genetics 16:405-436, each of

which is incorporated herein by reference).

Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleotide triphosphates (typically dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering systems. Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis. In some cases, the target regions may encode at least a portion of a protein expressed by the cell. In this instance, mRNA may be used for amplification of the target region. Alternatively, PCR can be used to generate a cDNA library from RNA for further amplification, the initial template for primer extension is RNA. Polymerizing agents suitable for synthesizing a complementary, copy-DNA (cDNA) sequence from the RNA template are reverse transcriptase (RT), such as avian myeloblastosis virus RT, Moloney murine leukemia virus RT, or Thermus thermophilus (Tth) DNA polymerase, a thermostable DNA polymerase with reverse transcriptase activity marketed by Perkin Elmer Cetus, Inc. Typically, the genomic RNA template is heat degraded during the first denaturation step after the initial reverse transcription step leaving only DNA template. Suitable polymerases for use with a DNA template include, for example, E. coli DNA polymerase I or its Klenow fragment, T4 DNA polymerase, Tth polymerase, and Taq polymerase, a heat-stable DNA polymerase isolated from Thermus aquaticus and commercially available from Perkin Elmer Cetus, Inc. The latter enzyme is widely used in the amplification and sequencing of nucleic acids. The reaction conditions for using Taq polymerase are known in the art and are described in Gelfand, 1989, PCR Technology, supra.

4. Allele Specific PCR

Allele-specific PCR differentiates between chromosome 7 target regions differing in the presence or absence of a variation or polymorphism. PCR amplification primers are chosen which bind only to certain alleles of the target sequence. Thus, for example, amplification products are generated from those chromosome 7 sets which contain the primer binding sequence, and no amplification products are generated in chromosome 7 sets without the primer binding sequence. This method is described by Gibbs, Nucleic Acid Res. 17:12427-2448 (1989).

5. Allele Specific Oligonucleotide Screening Methods

Further diagnostic screening methods employ the allele-specific oligonucleotide (ASO) screening methods, as described by Saiki et al., Nature 324:163-166 (1986). Oligonucleotides with one or more base pair mismatches are generated for any particular allele. ASO screening methods detect mismatches between variant target genomic or PCR amplified DNA and non-mutant oligonucleotides, showing decreased binding of the oligonucleotide relative to a mutant oligonucleotide. Oligonucleotide probes can be designed that under low stringency will bind to both polymorphic forms of the allele, but which at higher stringency, bind to the allele to which they correspond. Alternatively, stringency conditions can be devised in which an essentially binary response is obtained, i.e., an ASO corresponding to a variant form of the CYP11a1 gene will hybridize to that allele, and not to the wildtype allele.

6. Ligase Mediated Allele Detection Method

Target regions of a test subject's DNA can be compared with target regions in unaffected and affected family members by ligase-mediated allele detection. See Landegren et al., Science 241:1077-1080 (1988). Ligase may also be used to detect point mutations in the ligation amplification reaction described in Wu et al., Genomics 4:560-569 (1989). The ligation amplification reaction (LAR) utilizes amplification of specific DNA sequence using sequential rounds of template dependent ligation as described in Wu, supra, and Barany, Proc. Nat. Acad. Sci. 88:189-193 (1990).

7. Denaturing Gradient Gel Electrophoresis

Amplification products generated using the polymerase chain reaction can be analyzed by the use of denaturing gradient gel electrophoresis. Different alleles can be identified based on the different sequence-dependent melting properties and electrophoretic migration of DNA in solution. DNA molecules melt in segments, termed melting domains, under conditions of increased temperature or denaturation. Each melting domain melts cooperatively at a distinct, base-specific melting temperature (Tm). Melting domains are at least 20 base pairs in length, and may be up to several hundred base pairs in length.

Differentiation between alleles based on sequence specific melting domain differences can be assessed using polyacrylamide gel electrophoresis, as described in Chapter 7 of Erlich, ed., PCR Technology, Principles and Applications for DNA Amplification, W.H. Freeman and Co, New York (1992), the contents of which are hereby incorporated by reference.

Generally, a target region to be analyzed by denaturing gradient gel electrophoresis is amplified using PCR primers flanking the target region. The amplified PCR product is applied to a polyacrylamide gel with a linear denaturing gradient as described in Myers et al., Meth. Enzymol. 155:501-527 (1986), and Myers et al., in Genomic Analysis, A Practical Approach, K. Davies Ed. IRL Press Limited, Oxford, pp. 95-139 (1988), the contents of which are hereby incorporated by reference. The electrophoresis system is maintained at a temperature slightly below the Tm of the melting domains of the target sequences.

In an alternative method of denaturing gradient gel electrophoresis, the target sequences may be initially attached to a stretch of GC nucleotides, termed a GC clamp, as described in Chapter 7 of Erlich, supra. Preferably, at least 80% of the nucleotides in the GC clamp are either guanine or cytosine. Preferably, the GC clamp is at least 30 bases long. This method is particularly suited to target sequences with high Tm's.

Generally, the target region is amplified by the polymerase chain reaction as described above. One of the oligonucleotide PCR primers carries at its 5' end, the GC clamp region, at least 30 bases of the GC rich sequence, which is incorporated into the 5' end of the target region during amplification. The resulting amplified target region is run on an electrophoresis gel under denaturing gradient conditions as described above. DNA fragments differing by a single base change will migrate through the gel to different positions, which may be visualized by ethidium bromide staining.

8. Temperature Gradient Gel Electrophoresis

Temperature gradient gel electrophoresis (TGGE)is

based on the same underlying principles as denaturing gradient gel electrophoresis, except the denaturing gradient is produced by differences in temperature instead of differences in the concentration of a chemical denaturant. Standard TGGE utilizes an electrophoresis apparatus with a temperature gradient running along the electrophoresis path. As samples migrate through a gel with a uniform concentration of a chemical denaturant, they encounter increasing temperatures. An alternative method of TGGE, temporal temperature gradient gel electrophoresis (TTGE or tTGGE) uses a steadily increasing temperature of the entire electrophoresis gel to achieve the same result. As the samples migrate through the gel the temperature of the entire gel increases, leading the samples to encounter increasing temperature as they migrate through the gel. Preparation of samples, including PCR amplification with incorporation of a GC clamp, and visualization of products are the same as for denaturing gradient gel electrophoresis.

9. Single-Strand Conformation Polymorphism Analysis

Target sequences or alleles at the CYP11a1 locus can be differentiated using single-strand conformation polymorphism analysis, which identifies base differences by alteration in electrophoretic migration of single stranded PCR products, as described in Orita et al., Proc. Nat. Acad. Sci. 86:2766-2770 (1989). Amplified PCR products can be generated as described above, and heated or otherwise denatured, to form single stranded amplification products. Single-stranded nucleic acids may refold or form secondary structures which are partially dependent on the base sequence. Thus, electrophoretic mobility of single-stranded

amplification products can detect base-sequence difference between alleles or target sequences.

10. Chemical or Enzymatic Cleavage of Mismatches

Differences between target sequences can also be detected by differential chemical cleavage of mismatched base pairs, as described in Grompe et al., Am. J. Hum. Genet. 48:212-222 (1991). In another method, differences between target sequences can be detected by enzymatic cleavage of mismatched base pairs, as described in Nelson et al., Nature Genetics 4:11-18 (1993). Briefly, genetic material from a patient and an affected family member may be used to generate mismatch free heterohybrid DNA duplexes. As used herein, "heterohybrid" means a DNA duplex strand comprising one strand of DNA from one person, usually the patient, and a second DNA strand from another person, usually an affected or unaffected family member. Positive selection for heterohybrids free of mismatches allows determination of small insertions, deletions or other polymorphisms that may be associated with alterations in androgen metabolism.

11. Non-PCR Based DNA Diagnostics

The identification of a DNA sequence linked to CYP11a1 can be made without an amplification step, based on polymorphisms including restriction fragment length polymorphisms in a patient and a family member. Hybridization probes are generally oligonucleotides which bind through complementary base pairing to all or part of a target nucleic acid. Probes typically bind target sequences lacking complete complementarity with the probe sequence depending on the stringency of the hybridization conditions. The probes are preferably labeled directly or indirectly, such that by assaying

for the presence or absence of the probe, one can detect the presence or absence of the target sequence. Direct labeling methods include radioisotope labeling, such as with ^{32}P or ^{35}S. Indirect labeling methods include fluorescent tags, biotin complexes which may be bound to avidin or streptavidin, or peptide or protein tags. Visual detection methods include photoluminescents, Texas red, rhodamine and its derivatives, red leuco dye and 3, 3', 5, 5'-tetramethylbenzidine (TMB), fluorescein, and its derivatives, dansyl, umbelliferone and the like or with horse radish peroxidase, alkaline phosphatase and the like.

Hybridization probes include any nucleotide sequence capable of hybridizing to the porcine chromosome where CYP11a1 resides, and thus defining a genetic marker linked to CYP11a1, including a restriction fragment length polymorphism, a hypervariable region, repetitive element, or a variable number tandem repeat. Hybridization probes can be any gene or a suitable analog. Further suitable hybridization probes include exon fragments or portions of cDNAs or genes known to map to the relevant region of the chromosome.

Preferred tandem repeat hybridization probes for use according to the present invention are those that recognize a small number of fragments at a specific locus at high stringency hybridization conditions, or that recognize a larger number of fragments at that locus when the stringency conditions are lowered.

The following examples are provided to illustrate embodiments of the present invention. They are not intended to limit the invention in any way.

EXAMPLE I

A Genetic Marker for Meat Quality, Growth, Carcass and Reproductive Traits in Pigs

In accordance with the present invention, a genetic marker has been identified and characterized which is associated with improved meat quality and improved growth and carcass traits in pigs. The following materials and methods were utilized in the practice of Example I.

Testis tissue samples were obtained from fifty Yorkshire boars for which growth, carcass, and boar taint data had previously been collected. Boars were weaned at approximately 10 weeks of age, assigned to pens, and fed a standard grower-finisher diet to a final weight of approximately 120 kg. Boars were killed by electrical stunning and exsanguination at the Penn State University meats Laboratory. Testes, bulbourethral glands and submaxillary salivary glands were collected, trimmed, and weighed. Carcasses were weighed and then chilled overnight. The following day data were collected for standard carcass measurements such as carcass length, loin eye area, fat depth and marbling.

The assay for submaxillary salivary gland delta-16-androstenes was adapted from a procedure developed by Squires (J. Animal Sci. 69: 1092-1100, 1991). Briefly, submaxillary salivary glands were trimmed and minced in a food processor (Cusinart) and one gram of minced tissue was placed in a 50 ml test tube. Methanol (5 ml) was added and the mixture was homogenized for 30 sec by Polytron. Samples were placed in a centrifuge for 5 min @ 2800 rpm. Three ml of distilled water were added to 3 ml of the supernatant and mixed by vortexing. Six ml of hexane were added to extract the delta-16-androstenes. The mixture was vortexed and allowed to stand for 5 min

for the phases to separate. Three milliliters of the organic phase were transferred to a glass culture tube and the extract was dried under nitrogen while in a water bath (30°C). Color reagents were added (.5 ml of .5% resorcylaldehyde in glacial acetic acid plus .5 ml of 5% sulfuric acid in glacial acetic acid). The tubes were placed in a heat block for 10 min at 95 C. Development of a violet color, an index of the presence of delta-16-androstenes, was measured by pipetting 100 µl of the test solution into a well in a 96-well microplate. Absorbance was measured at several wavelengths near the known absorbance maximum for Δ16-androstenes (593 nm) and compared against standard test solutions containing 5α-androst-16-ene-3β-ol (the predominant Δ16-androstene in the submaxillary salivary gland). Concentration of Δ16-androstenes was established by generation of a standard curve with the standard test solutions.

Data were analyzed by ANOVA using the GLM procedures of SAS (1990).

Testis tissue samples were obtained from storage (-20°C) for ten boars: five that had the highest concentrations of Δ16-androstenes (high boar taint) and five that had the lowest concentrations of Δ16-androstenes (low boar taint). DNA was extracted by Proteinase K digestion. Approximately 50 mg of testis tissue was wrapped in aluminum foil and frozen in liquid nitrogen. The sample was then pulverized and approximately 20 mg was placed in a microfuge tube with .5 ml digestion buffer (50 mM Tris, pH 8.5; 1mM EDTA; 0.5% Tween 20; 200 µg/ml proteinase K (Gibco Life Technologlies, Grand Island, NY). Proteinase K was stored at -20°C in stock solution (20 mg/ml proteinase K; 1-mM Tris-HCl, pH 7.5; 20 mM calcium chloride, and 5% glycerol). The samples were suspended in digestion

buffer and placed in a water bath @ 55°C for 3 hours. Samples were centrifuged for 1 min @13,000 g and placed in a heat block for 10 min @ 95°C. Samples were removed and stored at -20°C until analyzed.

Four sets of primers were obtained which corresponded to approximately 600 bp each for a total of approximately 2.4kb of the 5'UTR of the porcine CYP11a1 gene (sequence obtained from Urban, et al., J. Biol. Chem. 269:25761-25769, 1994). See Figure 1. Polymerase Chain Reactions were initiated for each primer set for each of the ten DNA templates. PCR was performed as follows.

1. 2 min @ 94 C.
2. 1 min @ 94 C
3. 1 min @ 55 C
4. 1 min @ 72 C
5. 35 cycles to (2.)
6. 2 min @ 72 C
7. hold at 5 C

Reactions were performed using 10x buffer (w/$MgCl_2$); dNTP's (10 nmol); primer CYPscc For1 (20 pmol);primer CYPscc Rev1 (20 pmol); Taq polymerase ;ddH_2O and DNA template (1:10 dilution of Proteinase K digested sample, approximately 100 ng).

PCR products were analyzed by agarose gel electrophoresis, and the ~600 bp bands cut out of the agarose gel and purified using the QIAquick gel extraction kits (QIAGEN Inc., Valencia CA). The nucleotide sequences of each of the forty PCR products was determined in both forward and reverse directions using an ABI Prism Model 377 Sequencer (Perkin Elmer, CA) at the Penn State Nucleic Acid Facility, PSU Biotechnology Institute.

The sequences of the PCR products were aligned manually and examined for differences between the ten animals. While there were 37 differences in the samples when compared with the published sequence (Urban et al., 1994, *supra*), there was only one base pair that varied among this group of animals. At position -155 (155 bases before the start site ATG codon), six of the samples had the cytosine (CC), and four were polymorphic; that is they had both the cytosine and thymidine (CT), indicating heterozygosity at that base pair. Of significant interest was that all five of the high taint boar samples were the CC genotype, whereas four out of five of the low taint boar samples had the CT genotype.

This polymorphism was located in a restriction enzyme recognition site such that the presence of the T allele would change the restriction fragment length pattern observed after digestion with specific restriction enzymes. In this particular case, the restriction enzyme used was SphI (New England Biolabs). Presence or absence of the T allele in the DNA samples from the full group of fifty boars was determined by Restriction Fragment Length Polymorphism analysis involving examination of restriction digests of CYP11a1 5'UTR using SphI. For exemplary gel, see Figure 2. Presence of the T allele, either homozygous (TT) or heterozygous (CT) was associated with low boar taint. Presence of the CC allele was associated with high boar taint, as well as with increased testis weight, increased bulbolurethral gland length and weight, and increased submaxillary salivary gland weight. See Figure 3 and Table 4. In addition, boars that possessed the CC allele exhibited a 5.9% improvement in rate of gain, and had greater amounts of lean muscle as evidenced by longer carcasses, and tended to have less fat as determined by backfat depth measurements. Boars

with the CC allele also tended to have higher concentrations of serum testosterone in blood samples taken at slaughter.

A retrospective analysis of production records of direct female relatives (dams and siblings) of these boars revealed that those females related to boars possessing the T allele tended to have slightly larger litter sizes (+.31 pigs/litter) and weaned heavier litters (+4.27 kg). Thus this polymorphism appears to confer beneficial fertility and productivity traits to female pigs.

EXAMPLE II

A Genetic Marker for Meat Quality, Growth, Carcass and Reproductive Traits in Cows and Chickens

The identification and characterization of the CYP11a1 polymorphism in pigs facilitates the characterization of the corresponding polymorphism in bovines which are associated with improved reproductive and carcass traits. The bovine CYP11a1 sequence is provided in Figure 5. A suitable primer set for amplifying the bovine homologue of the 5' UTR for the CYP11a1 gene has the following sequences: Sense: 5'-GCAGATGTCCCTGGTGATTC-3'; and Antisense: 5'-TGAACGGAGGGGAAGCC-3'.

Amplified bovine CYP11a1 sequences and corresponding genetic traits are then characterized as set forth herein for the porcine CYP11a1 gene.

Figure 6 depicts the CYP11a1 gene from chicken. In order to assess genetic changes in a more lengthy 5'UTR sequence from the chicken CYP11a1 sequence provided in Genbank, the cDNA sequence provided in Figure 6 is utilized as the basis for 5' rapid amplification of cDNA ends (RACE) using a kit from Clontech containing RACE-

ready cDNA prepared from chicken. Clones obtained from this RACE approach yield 5' end points of the chicken CYP11a1 sequence for further analysis of genetic changes in the 5'UTR associated with improved reproductive and carcass traits. Genetic polymorphisms and alterations so identified are within the scope of the present invention. Suitable protocols for practicing RACE are provided in Current Protocols of Molecular Biology; J. Wiley & Sons, Inc. 1998, Chapter 15.6.9, the entire disclosure of which is incorporated by reference herein.

The present invention is not limited to the embodiments specifically described above, but is capable of variation and modification without departure from the scope of the appended claims.

Claims

What is claimed is:

1. A method of screening test subjects to identify those more likely to have better growth, development, reproduction and carcass traits such as rates of gain, carcass length, or litter size, comprising: obtaining a sample of genetic material from a test subject and assaying for the presence of a polymorphism in the CYP11a1 gene which is associated with rate of gain, carcass length, and litter size.

2. The method of claim 1 wherein said step of assaying is selected from the group consisting of restriction fragment length polymorphism (RFLP) analysis, heteroduplex analysis, single strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE).

3. The method of claim 1, wherein said step of assaying for the presence of said polymorphism comprises the steps of digesting said genetic material with a restriction enzyme that cleaves the CYP11a1 gene in at least one place; separating the fragments obtained from the said digestion; detecting a restriction pattern generated by said fragments; and comparing said pattern with a second restriction pattern for the CYP11a1 gene obtained by using said restriction enzyme, wherein said second restriction pattern is associated with increased rates of gain, increased carcass length, and increased litter size.

4. A method as claimed in claim 1, wherein said test subject is selected from the group consisting of pigs, cows and chickens.

5. The method of claim 3 wherein said restriction enzyme is SphI and said test subject is a pig.

6. The method of claim 3 wherein said separation is by gel electrophoresis.

7. The method of claim 3 wherein said step of comparing said restriction patterns comprises identifying specific fragments by size and comparing the sizes of said fragments.

8. The method of claim 5 further comprising the step of amplifying the amount of porcine CYP11a1 gene or a portion thereof which contains said polymorphism, prior to said digestion step.

9. The method of claim 3 wherein said restriction site is located in the untranslated region of the CYP11a1 gene.

10. The method of claim 7 wherein said amplification includes the steps of selecting a forward and a reverse sequence primer capable of amplifying a region of the porcine CYP11a1 gene which contains a polymorphic restriction site.

11. The method of claim 10 wherein said forward and reverse primers are between 10 and 50 nucleotides in length and selected from SEQ ID NO: 1.

12. The method of claim 10 wherein said forward primer is SEQ ID NO:2 and said reverse primer is SEQ ID NO:3.

13. The method of claim 6 wherein said step of

detecting sizes of said fragments comprises the steps of separating said fragments by size using gel electrophoresis in the presence of a control DNA fragment of known size; contacting said separated fragments with a probe that hybridizes with said fragments to form probe-fragment complexes; and determining the size of separated fragments by detecting the presence of the probe fragment.

14. A method for identifying a genetic marker for pig growth rate, carcass length, litter size, or boar taint comprising the steps of breeding male and female pigs of the same breed or breed cross or derived from similar genetic lineages; determining the growth rates, carcass lengths, number of offspring, or presence of boar taint; determining the presence of a polymorphism in the CYP11a1 gene of each pig; and associating the growth rate, carcass length, number of offspring, or presence of boar taint of each pig with said polymorphism thereby identifying a polymorphism for these traits.

15. The method of claim 14 further comprising the step of selecting pigs for breeding which are predicted to have better growth rates, longer carcasses, increased litter size, or decreased boar taint by said marker.

16. The method of claim 14 wherein said analysis comprises digestion of PCR amplified DNA with the restriction enzyme SphI.

17. The method of claim 12 wherein said polymorphism associated with growth rate, carcass length, litter size, or boar taint is detected by use of forward and reverse primers comprising at least 4

consecutive bases in SEQ NOS: 2 and 3.

18. A kit for evaluating a sample of porcine DNA comprising, in a container, a reagent that identifies a polymorphism in the porcine CYP11a1 gene.

19. The kit of claim 18 wherein said reagent is a primer that amplifies the porcine CYP11a1 gene or a fragment thereof.

20. The kit of claim 18 further comprising a DNA polymerase, a restriction enzyme which cleaves the porcine CYP11a1 gene in a least one place; and forward and reverse primers capable of amplifying a region of the porcine CYP11a1 gene which contains a polymorphic site.

21. A primer for assaying for the presence of a polymorphic SphI site in the porcine CYP11a1 gene wherein said primer comprises a sequence from the group of SEQ ID NO:2 and SEQ ID NO:3.

22. A genetic marker associated with growth rate, carcass length, litter size, and boar taint in pigs, said marker comprising a polymorphism in the porcine CYP11a1 gene.

23. The genetic marker of claim 22 wherein said polymorphism is a SphI restriction site.

24. The marker of claim 22 wherein said polymorphism is located in the 5' untranslated region of the porcine CYP11a1 gene.

25. A DNA sequence from the porcine CYP11a1 gene 5'

untranslated region, said sequence consisting of SEQ ID NO: 1.

26. A primer designed to amplify a polymorphic SphI restriction site in the porcine CYP11a1 gene wherein said primer is 4 or more continuous bases from SEQ ID NO: 1.

27. A primer designed to amplify a polymorphic SphI restriction site in the porcine CYP11a1 gene wherein said primer is a reverse primer generated from the SEQ ID NO: 1.

28. A method for screening pigs to determine those more likely have increased growth rates, longer carcasses, larger litters, higher boar taint, and/or those less likely to exhibit increased growth rates, longer carcasses, larger litters, or higher boar taint, which method comprises of the steps: determining the alleles of the CYP11a1 gene present in a pig; determining the alleles of other markers for genes know to affect growth rate, carcass length, litter size, or boar taint; and selecting for animals with favorable combinations of alleles and against those carrying unfavorable combinations.

29. The method of claim 28 wherein the determination of CYP11a1 alleles comprises determining the presence of at least one allele associated with at least one DNA marker linked either directly or indirectly to CYP11a1.

30. The method of claim 28 wherein the DNA marker is a microsatellite.

31. The method of claim 28 wherein the DNA marker is SO064, SO102, S0078, SO158, S0066, SW304, SW1083, SO101, or S0212.

32. The method of claim 28 wherein the marker is selected from the group of tumor necrosis factor alpha (TNFα), CYP11a1, prolactin (PRL), estrogen receptor (ER) and prolactin receptor (PRLR).

Figure 1.

SEQ ID NO:1

GCTCCAAAGAGACATTTTGGGGTGGCAAAATAGTCTACAGGATTCTATGGCATA
GGAGACAACTCTCAGATAGCTCTGCAGACCTGCTCCAAAGAAGTATAGGAGAAG
CCAGGATTTATAAGAACTTTTTTGTTGGGAAAATAAATGTAGTCAAACATAAAAAG
ACAACTGCTAATAACAAACAATAGACATGTCAAGATAATGACCTTAGTGCCTTTCT
ATGTGTGGAAAGACTCAAGAATCTGGGGTCATTGAACTTTTTCCTTAGATATGCA
TCTTAATATCCTGGGGTCAGTATAATCCAAATGCTTCCTGTTTTTCTCCATCCTAA
AGTCCCCTCCGGGTGCACTGATGGGTTCCCCTCCAGTGGGCAACTGCAGTGGC
AATTGGCTTGATCTCTGTAGAACTGGAATGGTGGGCAACATTCTTTTCTTTACAG
TATCCTGAGTCTGGGAGGGGCTGTGTGGGCCAGAGCCTGNATGCAGGAGGAG
GAGGGAGTCTGATCGCTTAGTCAGCTTCTCGCTTAACCTTGAGCTGGTGGTTAT
AAGCTGGGCCCCAGGCGCCCGAGGCCAGACTCACCTCATCAGGCCCTGCTGCA
GTGGGAGCAGGGAGAGTAGCAGTGGTAGGGGCAGCATG

N = C or T at polymorphic site

SEQ ID NO:2

Forward primer:

GCTCCAAAGAGACATTTTGGGGTGGC

SEQ ID NO:3

Reverse primer:

CATGCTGCCCCTACCACTGCTACTCT

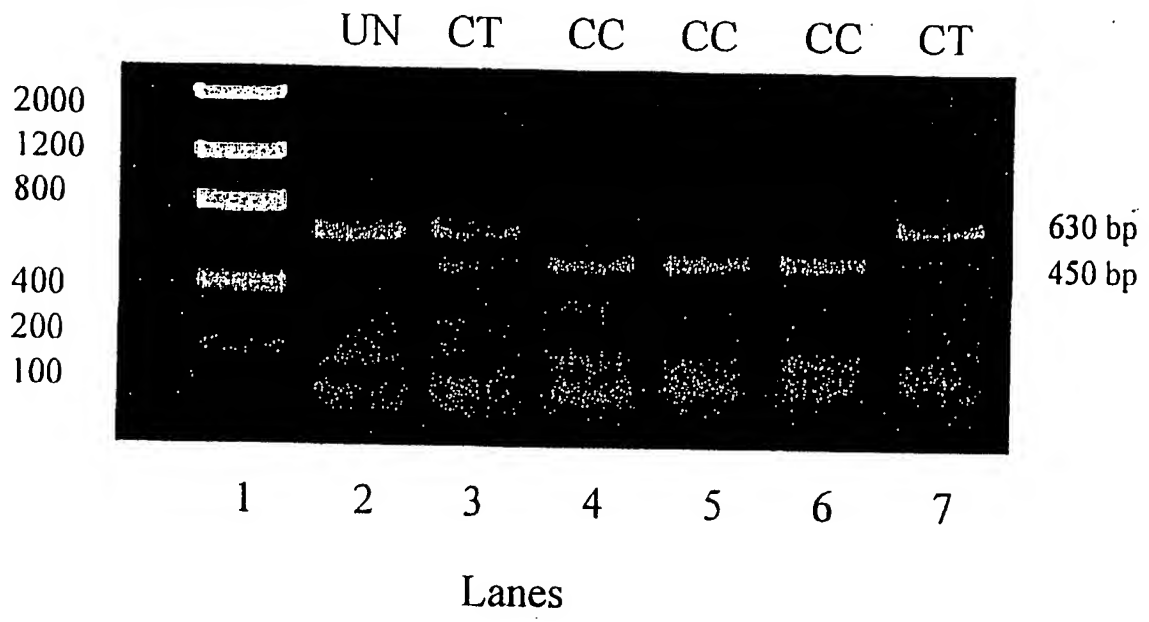
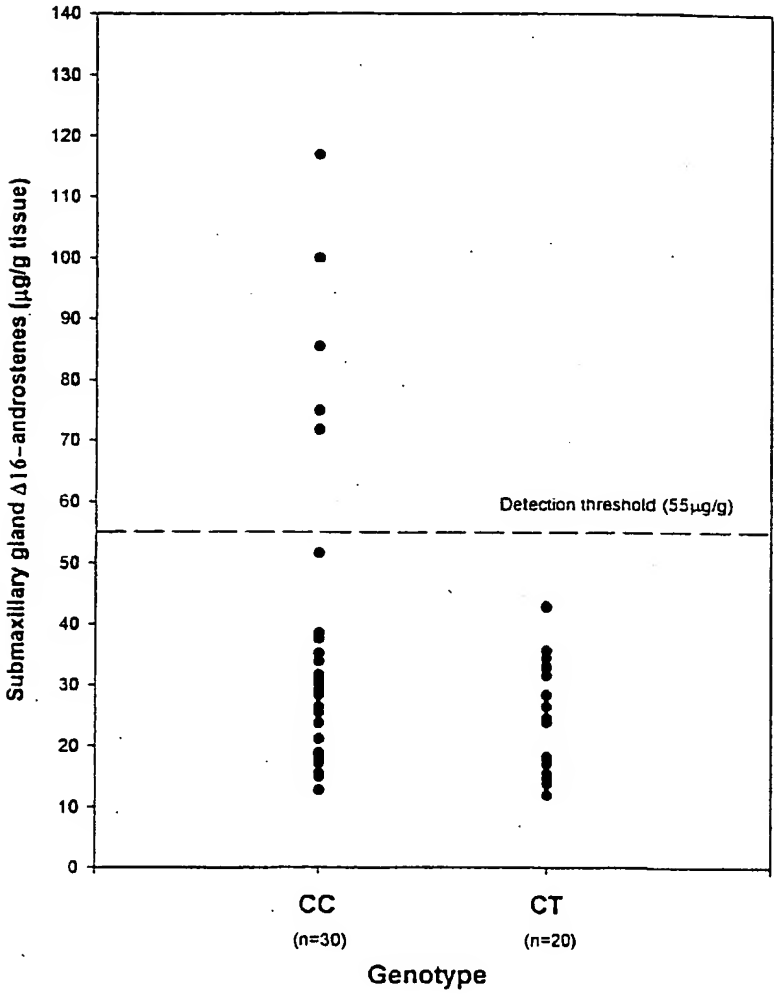


Figure 2. SphI restriction digest of porcine CYP11a1 PCR fragment

Figure 3. Comparison of submaxillary salivary gland Δ16-androstenes in boars possessing a CYP11a1 single nucleotide polymorphism.



	Genotype		
	CC	CT	P value
Rate of gain (kg BW/d)	0.76 ± .01	0.72 ± .01	.05
Carcass length (cm)	85.17 ± .38	82.96 ± .47	.001
Submaxillary salivary gland (SMG) wt (g)	92.1 ± 3.1	71.5 ± 4.9	.0001
Δ16-androstenes in SMG (μg/g)	38.7 ± 4.1	23.9 ± 5.0	.05
Relative SMG wt (g/kg BW)	0.72 ± .023	0.58 ± .027	.001
Bulbourethral gland length (mm)	128.8 ± 2.4	117.7 ± 2.9	.01
Relative bulbourethral gland wt (g/kg BW)	93.8 ± 4.0	73.5 ± 4.9	.01
Testis wt (g)	628.6 ± 27.1	530.2 ± 25.4	.05
Relative testis wt (g/kg BW)	4.92 ± .20	4.33 ± .24	.10
Serum testosterone at slaughter (ng/ml)	2.04 ± .28	1.59 ± .35	.32

Figure 4. Growth, carcass, and reproductive traits of pigs with CC or CT CYP11a1 polymorphism.

```
   1 gcagatgtcc ctggtgattc ctgaaacagg ccctctgttt aaattcttca gcagttagag
  61 ggaaggtcaa tttttcccaa ggcttttggg ctttgattgt tttcattttt aaattatctg
 121 cattctaaag agatattttg ggtggcagat tttgctctcc tacaggactt tgtctaggag
 181 acggctctca ggccagctcc gacgactgtt ccaaagaagt aagggaaagc tagggtttat
 241 atcaatcttt ttttttgctg ggagaagggg gatgaacatg tagtcaaaca taaaaagatc
 301 actgctaatc ccaaacaaca gacacctcaa gtgaatggtt ttagtgtttt tctatatatg
 361 ttgtttagtc actaagtcct gtccgactct tttgcgactc catagactgt agcccaccaa
 421 gctcctctgt ccatgggatt tttctaggca agaatactgg agtgggttgc catttccttc
 481 tccctgggat cttcctaacc caaggactga acccttgtct cctgcattgc aggtggattt
 541 tttaccgact gagccaccag ggaagttatg tgtgcaagaa tccggggtca tggaaatttt
 601 cccttagata tacatcgtat ctagggacca gtacaatgca aatgcttcct gtttttcttc
 661 atcctgaagt ctcctcaggg tgcattgagg gagggagtcc cctcaggtgg gtgaccacag
 721 tggctgacgc ttgatgttgt agaactggaa tgatgggtta cattctttcg tttacagtac
 781 tgagtctggg aggagctgtg tgggctggag tcagccggag gaggctgacc gccctgtcag
 841 cttctcactt agccttgagc tggtgattat aagctgggtc ccagggtccc agggccagag
 901 tcacctgctg cagtacgagc agagacagca gcagctgtgg gggcagcatg ctagcaaggg
 961 ggcttccgct ccgttcagcc ctggtcaaag cctgcccacc catcctgagc tcagtggggg
1021 agggctgggg ccaccacagg gtgggcactg gagagggagc tggcatctcc acaaagaccc
1081 ctcgccccta cagtgagatc ccctcccctg gtgacaatgg ctggcttaac ctctaccatt
1141 tctggaggga gaagggctca cagagaatcc actttcgcca catcgagaac ttccagaagt
1201 atggccccat ttacaggtaa gcctggcagg aggattgggg ctggcgggat agggaagcct
1261 gtggtggccc cctccctgaa aggtctgccc tccccttcca ggctctggtt cacctctgac
1321 tttatttctt cctgcctggc ggtggcagga gtagagttaa tgcttcccag acagtgggtt
1381 cacttcccag ccctgaggcc tcaacagtcc ccgggctcta cacccttaga aactttgggg
1441 aggtggggag gcccaagaaa ataagccccg g
```

FIGURE 5

```
   1 ctttttcgg ttgtaccttt gtctctgtac agatattttg taatatatta aaaacaaaac
  61 ctactgagct cctcgccttg agcccaggat tcagggataa gagcgaggtc gccccggccg
 121 tgcgccgccc tgctcccatg ctctccaggg ctgcacccat agcgggcagc tttcaggcat
 181 gccgctgtgc cggagggatc ccagccctcg cgggggtcca ctacccattg cccagctcct
 241 cgggagctcg gcctttcgac caggtgccgg gtgaatggag agcgggttgg ctcaacctgt
 301 accacttctg gaaggaggga ggcttccaca acgtgcacaa catcatggcc agcaagttcc
 361 agcgctttgg gcccatctac agggagaagt tgggtgtcta cgagagcgtg aatatcatca
 421 gcccccgcga tgcggccacg ctcttcaagt cagaggggat gctgcccgag cgcttcagcg
 481 tgcccccatg ggtggcatac cgtgactacc gcaacaagcc ctacggcgtg ctcctcaaga
 541 caggggaggc ctggcgctcg gaccgcctga ccctgaacaa ggaggtgctg tcgccgcagg
 601 tggtggacag cttcgtgccc ttgctggacc aggtgagcca ggacttttg cggcgggcac
 661 gggcgcaggt ccagcagagc ggccgggagc gctggacggc cgacttcagc cacgagctct
 721 tccgctttgc cttggagtct gtgtgccacg tgctgtatgg ggaacgcctg gggctgctgc
 781 aggactttgt ggacccagag gcacagcagt tcatcgacgc cgtcaccctc atgttccaca
 841 ccacctcccc catgctctac gtgccacccg ccctgctccg ccacctcaac accaagacat
 901 ggcgtgacca cgtgcatgct tgggatgcca tcttcacaca ggctgacaaa tgtatccaaa
 961 acgtttaccg ggacatccgg ctgcaacgca agagcaccga ggagcacacg ggcatcctct
1021 tcagcctcct tgtgcaggac aagctgcccc tggatgacat caaggccagc gtcaccgaga
1081 tgatggcggg cggcgtggac acgacttcca tgactctgca atgggccatg ctggagctgg
1141 cacgatcccc gggcatccag gagcggctgc ggcagaggt gctggcagcc aagcaggagg
1201 cacaggggga cagggtgaag atgctgaaga gcatccgact gctcaaagcc gccatcaagg
1261 agactctcag gctgcacccg gtggcggtga cgctgcagag gtacaccaca caggaggtca
1321 tcctgcagga ctaccgcatc ccccccaaga cgctggtgca ggttggtctc tacgccatgg
1381 gacgagaccc tgaggtcttc cccaagccgg agcagttcaa ccctgagcgc tggctggtga
1441 tgggctccaa gcacttcaag ggactgagct ttgggtttgg gccacggcag tgtctgggtc
1501 gtcgcatcgc cgagctggag atgcagctct tcctcatgca catcctggag aactttaaga
1561 tcgaaaccaa gcgggcggtg gaagttggga ccaagttcga cctcattctt gtccctgaaa
1621 aacccatcta cctgagactg cggcccctcc agccccagga gtgacatggg gtgtccccag
1681 ttggtcccag cttggggaca cctccatcag ctcagcgcat tcagccttgg ctccagccct
1741 tcttacgcca tgggggagat ggctgccccc ttcccatttt cttcgcctct gatttgctct
1801 gtaatttctg caccaaaagc
```

FIGURE 6

INTERNATIONAL SEARCH REPORT

Internat al application No. .
PCT/US00/13168

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) :C07H 21/04; C12Q 1/68
US CL :435/6; 536/23.1, 24.33
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99/18192 A1 (THE PENN STATE RESEARCH FOUNDATION) 15 April 1999, see entire document.	1-32
A	NOLAN et al. Genotype of the P450scc locus determines differences in the amount of P450scc protein and maximal testosterone production in mouse Leydig cells. Mol. Endocrinol. October 1990. Vol. 4, No. 10, pages 1459-1464, see entire document.	1-32
A	DUROCHER et al. Genetic linkage mapping of the CYP11a1 gene encoding the cholesterol side-chain cleavage P450scc close to the CYP1a1 gene and D15S204 in the chromosome 15q22.33-q23 region. Pharamacogenetics. February 1998, Vol. 8, No. 1, pages 49-53, see entire document.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search 10 AUGUST 2000	Date of mailing of the international search report 05 SEP 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ROBERT SCHWARTZMAN Telephone No. (703) 305-0196

Form PCT/ISA/210 (second sheet) (July 1998) *

INTERNATIONAL SEARCH REPORT

Internat. .l application No.
PCT/US00/13168

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	DAVIS et al. Association of cytochrome b5 with 16-androstene steroid synthesis in the testis and accumulatioin in the fat of male pigs. J. Anim. Sci. May 1999, Vol. 77, No. 5, pages 1230-1235, see entire document.	1-32

INTERNATIONAL SEARCH REPORT

Internat. al application No.
PCT/US00/13168

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons: .

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 11, 12, 17, 21, 25-27
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

The claims are drawn to specific SEQ ID NOS but the claims could not be searched as the computer readable form of the Sequence Listing filed July 27, 2000 was blank (See attached CRF Problem Report).

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internat...al application No.
PCT/US00/13168

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

STN: Medline Biosis Embase CAPlus
WEST
Search Terms: boar taint, boar odor, CYP11a1, cytochrome P450, polymorphism